

MICROBIAL CELL RECOVERY FROM SOLID MATERIALS

(Mod. 3)

VOLUME I

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I. SUMMARY OF DEVELOPMENT OF RECOVERY METHODS

Microorganisms entrapped within solids constitute unique contamination control problems. Bacterial spores encased in plaster of paris, asbestos plastic cement, various plastic potting materials, and solid rocket propellants are more resistant to dry heat than those residing on surfaces (ref. 1). Due to this increased resistance to the effects of dry heat it was deemed necessary to develop assay procedures which would allow the detection of very small populations of microorganisms within the solids. Some solids within the spacecraft may be subjected to temperatures in excess of sterilization requirements and thus constitute a minimum contamination control problem. There are, however, solid components which are adversely affected by such treatment. It is essential that the microbial population within these solid materials be accurately known in order to assess their contribution to the total contamination load of the entire spacecraft.

Not only is it essential to develop adequate pulverization techniques for exposing these micro-organisms, but it is also necessary to develop methodology for overcoming the adverse environments provided by the spacecraft solids. At the time of fabrication of spacecraft components, microorganisms from the environment may exist and be incorporated as spores or as vegetative cells in any of a wide variety of physiological states. Obviously, the effects of various plastics, propellants, and other solids on the physiological states of microorganisms are important in the development of suitable recovery techniques. The ultimate aim of pulverization is to reduce the particle size to that which will statistically provide for optimum diffusion of nutrients into the microorganisms and thereby permit the outgrowth of these cells from their encased sites within the solid particle. Since bacterial and fungal spores are best adapted for survival in the internal regions of solids, the pulverization process must be optimized for their detection and cultural recovery. In the course of this pulverization, large microorganisms including fungal spores, mycelia, and large vegetative cells may be physically damaged. Thus, in order to assure recovery of such large cells, additional samples of each of the solids should

be pulverized in a manner modified for the recovery of large cells.

A wide range of substances are present within solids which interfere with the growth of microorganisms. Some plastic solids are polymerized through the use of free radical initiators. These initiators, or their products, may be present in minute but detectable quantities in some solids and must be neutralized in order to provide optimal recovery conditions. Solid rocket propellant contains large quantities of inhibitory substances, which must be leached from the pulverized propellant and neutralized prior to the application of culture techniques. Epoxy resins, coating materials, potting compounds and adhesives contain residual epoxide groups. Such functional groups must be reacted to prevent their interference with recovery of microorganisms.

II. INTRODUCTION

This final report on SN-42 (JPL Contract No. 950740) constitutes the pertinent data obtained in our investigations on the development of methods for the recovery of microorganisms from the interior of solid materials. In order to provide a high degree of reliability of microbial decontamination, it is essential that methods for the detection of viable microorganisms be developed and that these be as sensitive and reliable as possible. In the first phase of this program, under the direction of Dr. John Opfell, the investigation of a variety of methods for detecting the presence of viable microorganisms within solid materials were studied. These methods included culturing, electron spin resonance, electrophoresis, nonfluorescent staining, fluorescent staining, and autoradiography. As a result of these studies conducted in the application of the above methods, it became clear that with the exception of rather limited usefulness of fluorescent staining techniques, culturing represents the most useful, sensitive and easily applied method.

Electron spin resonance spectrometry involves the absorption of incident energy (usually in the microwave region) by unpaired electrons under the influence of a magnetic field. Electron spin resonance measures the magnetic moments of unpaired electrons and are somewhat analogous to the vibrational-rotational transitions in other forms of spectroscopy. Because free radicals occur in the process of metabolic activities within cells, this technique was studied to determine its potential usefulness as an independent means of assessing the presence of microbial contamination in solid materials. Indeed, it was found that differences existed between viable and nonviable microbial cells. Unfortunately, the order of magnitude of such differences is not sufficient to differentiate between other free radicals which may occur in a variety of nonliving solid materials (both organic and inorganic) of a wide variety of types. Since free radicals cannot be distinguished from those generated by small numbers of living microorganisms and inanimate solid particulates, this method was not considered to be of sufficient usefulness to warrant further investigation.

The cell walls of microorganisms contain chemical functional groups which vary in the nature and distribution of electrical charges. A wide variety of

microbial stains adhere strongly to the cell walls, and in some cases to the cytoplasmic constituents of microbial cells. The specificity and the degree of such staining is dependent upon the nature and distribution of the charge groups within the cell wall and the cytoplasm. In the application of this tool for the detection of microorganisms in solids, it is essential that the microorganism exposed from the solid be easily differentiated by microscopy from the debris. Not only is this of importance to determine the presence of microorganisms, but it is of greater usefulness to determine whether these microorganisms are viable or nonviable. Spores of B. subtilis var. niger, Clostridium sporogenes, and Ulocladium were obtained and the viability assessed by cultural methods. An aliquot of each of these microorganisms was subjected to the killing effects of dry heat, autoclaving, exposure to ethylene oxide, formaldehyde, and chlorine gas. Subsequently, both the viable and the nonviable organisms were stained and examined microscopically. No differences were noted that would permit the differentiation between viable and nonviable microorganisms. This method was abandoned.

A number of fluorescent dyes bind strongly to microorganisms. When these microorganisms are examined under a microscope equipped with suitable optics, the background may be adjusted to a nearly uniform black while the outlines of the microorganisms present a bright fluorescence. Thus, it would appear that this technique would be more useful than the nonfluorescent staining techniques since there are fewer interfering and confusing structures in the background. This method is only useful when the solid material and the other debris from the pulverized solid did not possess chemically charged groups which bind the dye. In the latter case the solid as well as the microorganism would show fluorescence and the method would be useless. Likewise it is necessary that the solid not be fluorescent. Under the experimental conditions of comparing viable and nonviable microorganisms, it was not possible to correlate the degree of fluorescence with viability. Thus this method suffers a severe deficiency. In addition, it is an exceedingly slow and painstaking technique which requires a high degree of standardization in order to use for the detection of microorganisms. The fundamental reason for abandoning this method was the lack of reliability in distinguishing viable cells from nonviable cells.

← This material would make a good case for culturing only as proof of viability - if it is indeed ever needed.

The incorporation of radioactively tagged beta emitting isotopes provide a means of determining potential cellular viability. The presence of the tagged metabolite may then be determined by the use of radioautography. The latter technique utilizes a sensitive fine grained photographic emulsion which is sensitive to weak beta emitting radiation. These weak beta rays interact with the photographic emulsion to provide opaque silver grains in a manner somewhat analogous to the effect of light on photographic emulsion. The presence of grain patterns which are confined within the outlines of the microbial cells, or fragments thereof, would provide evidence of viability, assuming that adequate controls are included. When the opaque silver grained pattern develops in regions outside the cell wall area, it may be possibly due to chemabsorption of the radioactive metabolite by the solid or inadequate washing. The resolution of this method is highest when the weakest forms of radiation capable of activating the silver particles in the emulsion is utilized. Maximum resolution depends upon a minimum distance between the microorganism containing the radioactive metabolite and the photographic emulsion. When spores of B. subtilis var. niger were incubated with solutions containing tritium labeled thymidine no radioactivity was detected in the radioautographs. This was because DNA is only duplicated during cell division which does not take place within spores.

Culturing represents the most reliable method thus far devised for the detection of viable microorganisms. This method depends on the ability of the microorganisms to undergo cellular division. There are, of course, deficiencies in the use of this method since physical and/or chemical trauma associated with the exposure of the microorganism from its location within the solid may result in a lack of ability to demonstrate the organisms' viability by culture methods. Thus, culture methods have long been established as useful techniques for the detection of viable microorganisms, and therefore, were used in Phases 2, 3, and 4.

Not only is it essential to develop adequate pulverization techniques for exposing these microorganisms, but it is also necessary to develop methodology for overcoming the adverse environments provided by the spacecraft solids. At the time of fabrication of spacecraft components, microorganisms from the environment may exist and be incorporated as spores or as vegetative cells in any of a wide variety of physiological states. Obviously, the effects of various plastics, propellants, and other solids on the physiological states of microorganisms

are important in the development of suitable recovery techniques. The ultimate aim of pulverization is to reduce the particle size to that which will statistically provide for optimum diffusion of nutrients into the microorganisms and thereby permit the outgrowth of these cells from their encased sites within the solid particle. Since bacterial and fungal spores are best adapted for survival in the internal regions of solids, the pulverization process must be optimized for their detection and cultural recovery. In the course of this pulverization, large microorganisms including fungal spores, mycelia, and large vegetative cells may be physically damaged. Thus, in order to assure recovery of such large cells, additional samples of each of the solids should be pulverized in a manner modified for the recovery of large cells.

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III. METHODS AND RESULTS

A. Pulverization

The pulverization of solid materials used in the fabrication of spacecraft may be accomplished through the use of high speed blenders, grills, mortar and pestle, ball and vortex mills, abrasive devices, and saws. (See references 2 to 9). These methods, each have their characteristic advantages and disadvantages. Control of the extent and uniformity of pulverization with these methods is difficult and in some cases not possible. The production of aerosols limits the usefulness of the high speed blenders and drilling techniques. With certain types of solids, explosions occur when the level of pulverization reaches a certain particle distribution range. The recovery of micro-organisms pulverized in solids using high speed blenders and drills was disappointingly low. It would appear likely that the lack of adequate control of heat generated in these forms of pulverization as well as the physical trauma incident to these methods may be responsible for such results. Sawing permits the control of both the heat production (by controlling the sawing rate) and the extent of pulverization through selection of the ideal tooth design.

Various blade and tooth confirmations produce widely different forms of pulverization. The types of saw blades used in the sawing of the various types of materials are numerous; however, the major parameters of importance in determining the types of pulverization produced are dependent upon the following factors; 1) tooth confirmation and design, 2) pitch (number) and size of teeth, 3) sawing rate, 4) pressure applied to the solid.

The most important factor affecting the effectiveness of pulverization using saw blades can be related to the design and configuration of the teeth. The angles of the tooth face, back, clearance and rake, in addition to the tooth spacing and go up depth are illustrated in Figure 1. There are several possible combinations of teeth configurations which produce widely different results in the pulverization of solids. The short nearly vertical angle tooth design typical of hacksaw blades appears to be the most useful for the

pulverization of rigid plastic solids.

There are a variety of ways in which the teeth are set (Figure 2). The alternate set teeth have one tooth to the right and the next to the left. This type of set is common for some types of plastic cutting blades and for nearly all wood cutting blades. The configuration has not proven to be effective in the pulverization of solids, since it produces large cuboidal chips. By adding one straight tooth between the pairs of right and left teeth, the type of chips that are produced differ. If the angle of the cutting blade is acute in respect to the surface of the solid, a ribbon is produced in place of a chip.

The raker set, which interposes a straight tooth between the left and right set of teeth with the hacksaw configuration, is capable of clearing the ribbon and partially disrupting it. The most common blade, containing some 22 to 32 teeth per inch, employs wavy set teeth. These teeth are formed by alternately bringing two or more teeth to the right and to the left in graduated degrees. In this configuration, a center line drawn through the center of each tooth would generate a smooth low pitch wavy curve. The blades with 32 teeth per inch were generally gang set. In this configuration the tooth edge portion of the blade was formed with a device which gang sets 6 to 8 teeth in one direction and alternates the set of the succeeding group of 8 teeth so as to form a wavy pattern throughout the entire length of the blade. These blades are designed for cutting. Among the commercially available blades, the fine tooth blades show advantages over the alternate or raker type configuration in pulverization of solids.

In spite of the fact that hacksaw blades are designed for cutting hard metals, it is desirable to use nickel molybdenum alloy blades or tungsten steel blades in order to minimize the rate of dulling on the blade. Dulling which occurs when using other types of hardened steel alloys may result in poor pulverization. Cross linked epoxy polymers with fillers cause surprisingly rapid dulling of blades. The types of blades studied

included a representative range of metal alloys.

The pressure applied to the blade during the sawing processes is of importance. Ideally this pressure should not exceed 100 grams per inch. The greater the amount of weight on the blades, the thicker the resulting ribbon. The length of blade is of significance since it is convenient to use slow long strokes which utilize the entire length of the blade and thus allow a more even continuous stroke and increase the uniformity of pulverization. The effective pressure vs. blade design for the pulverization of plastic materials studied is shown in Tables 1a to 1c.

Solid rocket propellant is the most difficult spacecraft component material to pulverize. The Dremel drill, slicing with the microtome and other methods which were studied were ineffective because of the peculiar characteristics in this substance (ref. 4). Sawing with appropriate types of hacksaw blades provided the most efficient pulverization. The particles produced were sticky and tended to agglomerate. However, the individual particles even though agglomerated in the culture media provided sufficient surface area for effective recovery of microorganisms (5). The particles produced by sawing differ depending upon the amount of pressure applied and the rate of sawing. Ribbon-like structures produced when the sawing pressure was less than 100 grams per inch were less than 15 microns in diameter and displayed a high instance of micro cracks and imperfections radiating in all directions, Figure 3. Care must be taken that the particles are collected and handled in such a way that agglomeration of particles is minimized. The typical microscopic appearance of this material is shown in Figures 4 and 5.

Polyester and epoxy resins are satisfactorily pulverized by sawing. Ribbons are produced whose thicknesses are generally 5 to 15 microns when a minimum amount of pressure (less than a 100 grams per inch) is applied by the blade to the plastic. The rigid highly cross-linked or filled rigid plastic tend to pulverize as a powder. The less rigidly cross-linked plastics and semi-flexible types readily form ribbons (Figures 6 to 9).

If we assume that a microbial spore in an idealized model will occupy a space of one cubic micron, in the absence of trauma, toxicity, and neglecting die away, the solid when pulverized to this size range should provide a theoretical recovery of 100 percent. If we further assume that the model for the solid particle is two microns on each side, the volume is increased eight-fold (Figure 10). Is it reasonable to suppose that the theoretical recovery is decreased by a similar ratio of volume increase. The worst case would require that the spore exist in the center of all planes within the cube. Under these conditions, the maximum distance between the spore and the exterior would be one half micron. The probability of this worst case would approach zero. The physical constraints of a particle containing the entrapped spore would be less than the theoretical cube case within any particle of two microns or less actually observed in the pulverized sample.

The worst case situation in such a particle would require that there be no micro cracks or imperfections in any plane which would encroach upon the central location of the spore. The probability of this occurring also approaches zero. Particles resulting from the pulverization showing a maximum dimension in any plane are generally sized by that dimension. Nearly all of the particles, with the exception in the longest plane, may be a small fraction of 5 microns. Statistically, nearly all such particles would certainly have, in one plane or another, distances much less than 5 microns. In the course of our studies on pulverization, we have observed that well over 80% of such classified particles contain cross-sectional distances of less than 2 microns. It might be noted that in Table Ic, when using a 32 tooth blade and light pressure, as much as 70% of the particles were less than 5 microns in size.

This material proves the need for ^{various} work on recovery methods

B. Leaching and Neutralization

Often soluble toxic substances may be extracted from pulverized solid materials. In some instances, simple dilution of the inhibitory substance is sufficient to allow nearly maximum growth rates in a reasonable final culture volume. Leaching may in some instances be necessary when inhibitors or toxic materials are not neutralizable. An important category of leachable inhibitors are polymerization, cross-linking agents, and catalysts. Solid plastic materials are polymerized by a variety of agents. Examples are benzoyl or isobutryl peroxide, epoxides, formaldehyde, or epichlorhydrin. The neutralization of benzoyl and isobutryl peroxide are summarized in Table 2. Of the various reactants studied, glutathione has proven to be the most effective and the least toxic. The other reductants investigated included sodium borohydride and cysteine.

While all these reductants were effective in the adjustment of the redox potential, it was found that cysteine sulfoxide was formed as a reaction product. This compound was toxic to some microorganisms. In addition, cysteine reacts with SS bonds within proteins. This amino acid can also react with transition state metals and thus interfere with trace metal metabolism of microorganisms when this amino acid is used in moderate to high concentrations. Borohydride is toxic to microorganisms largely due to its reaction product, borate ion. So in spite of its effectiveness in the adjustment of redox potential when used in dilute solutions, its slight toxicity limits its usefulness.

Virtually all epoxides and trimethylene oxides as well as tetramethylene oxides may be used in the synthesis of plastic materials. Tetrahydrofurane will polymerize readily only when cationic catalysts are used. Each of the above compounds show evidence of various degrees of toxicity. The epoxides are most toxic to both vegetative cells and spores. Tetrahydrofurane displays far less toxicity, and in many instances no toxicity at all.

The most important class of plastic materials utilized in potting compounds, electronic components, and encasement materials are the epoxy resins. A wide range of physical characteristics may be obtained with these compounds depending upon the types of monomers utilized and the nature and composition of the hardeners. A typical type of epoxy resin may be prepared by the base induced condensation of a polyhydroxy compound, usually a bisphenol, and utilizing in most cases epichlorhydrin which forms an intermediate of low molecular weight. This compound is essentially a linear polymer, containing terminal epoxide groups and pendant hydroxyls, Figure 11. An excess of epichlorhydrin in the reaction results in the termination of the polymeric chains with epoxy groups. These unreacted epoxide groups cause most of the toxicity associated with epoxy resins. The resins may be reacted to achieve maximum cross-linking by using curing or cross-linking agents, examples of which are amines and carboxylic acids and their anhydrides. In nearly all of the currently marketed epoxy resins epichlorhydrin is used.

Among the various methods of neutralizing this toxicity which were devised in our laboratory are 1) adjustment of pH to a moderately acidic concentration (pH 3 to 4) to open the epoxide ring or 2) reacting the epoxide with a suitable compound to form a chemical derivative. Both of these approaches were investigated and the results are summarized in Table 3. The reaction of epoxides with proteins is complex and must require some elaboration. It was discovered that this interaction at physiological pH's involved chiefly the amino acid histidine.

Epichlorhydrin was allowed to react with 2% casein hydrolysate for a period of 4 hours at 25°C. The amino acid which reacted most vigorously at pH 7.0 was histidine. The amino groups of some of the other amino acids were also reactive. The extent of reaction of the various amino acids in the mixture are summarized in Table 4. Epoxides may react with nucleic acids. The heterocyclic portion of the molecule may also react; however, it is of less importance. Epichlorhydrin exerts its more profound biological effects on DNA by causing either intramolecular cross-linking, cross-linking with

neighboring functional groups within the molecule, or with the nucleohistone present in the vicinity of the DNA or intramolecular cross-linking. The various forms of interaction are summarized in Figure 12. Nucleic acids were active in the neutralization of epichlorhydrin.

Beef extract (Difco Corporation) and RNA at 5% concentrations were effective neutralizers for epichlorhydrin. These substances form a viscous suspension at 5% concentrations. Higher concentrations of these neutralizers result in markedly inhomogeneous suspensions and do not improve the effectiveness of the neutralization (Table 5).

Leaching procedures were applied to Eccocoat 1C2 in an effort to improve the recovery from this solid (Table 6). The most promising leach fluids were ethylene chloride, chloroform, and trichlorotrifluoroethane. Slightly higher recoveries were obtained at 5°C than at 25°C. Since the order of magnitude of recovery remained low with the leaching procedures attempted, we abandoned leaching methods for Eccocoat 1C2 and Epon 901. Recoveries were sufficiently high for Stycast, Paraplast, Parlodion and Maraset compared to other data (4) not to apply leaching techniques. Leaching is used together with neutralization for solid rocket propellant (5).

Ultrasound at maximum output was useful in increasing the recovery from Stycast 1090 (Table 7).

C. Recovery of Microorganisms from Solids

All of the test organism types were recovered from Paraplast (Table 8). The vegetative cells were recovered from the latter solid only. Lower recoveries were obtained from Eccocoat 1C2, Epon 901, and RTV 40. Methods used were outlined in the protocol.

D. Culture Media

A variety of culture media including Bacto Anaerobic Agar*, Bacto Blood Agar Base, Thioglycollate*, Trypticase Soy**, were studied during the course of the past two years for the recovery of microorganisms from solids. The medium shown on Table 9 has proven to be the most effective. Although the termination of

*Difco Laboratories, Detroit, Michigan

** Baltimore Biological Laboratories

dormancy and the initiation of germination do not require special steps with the spores specified in this study, ingredients were incorporated in the growth medium to optimize these phenomenon, (tetrahydrofurane, alanine and casamino acids).

IV. FINAL PROTOCOL FOR THE CULTURING OF MICROORGANISMS FROM THE INTERIOR OF SOLID MATERIALS

A. Assembly of Sterile Chamber

A rectangular container (width 50 cm, length 100 cm having a capacity of 75 liters) is prepared utilizing 4-8 mil polyethylene tubing. A gland is provided which allows for exhaustion of the ethylene oxide and subsequent refilling with air. An aluminum frame and base supports the structure and provides a surface for the attachment of a vise. The contents of the chamber include ethylene oxide, the hacksaw assembly, liquid media, the sterile solutions of neutralizers, sterile standardized acid and base, and the solid materials to be studied. Several additional culture vessels are included which are utilized as controls by inference to demonstrate that the environment inside of the bag is sterile (Table 10).

Following the assembly of the contents of the bag, it is sealed utilizing a double heat seal seam. The bag is tested to determine whether leaks are present using a thermal conductivity cell of the gas chromatograph as a detector of leakage.

B. Surface Decontamination with Sterilant Gas

Evaporation of a previously determined volume of ethylene oxide in sufficient excess to maintain a concentration of 500 mg/L of space is allowed to take place within the bag at room temperature for a period of 12-16 hours. The sterilant gas mixture employed consists of 12% ethylene oxide and 88% Freon-12 or pure ethylene oxide.

Six test tubes each containing 10^2 spores of B. subtilis var niger are used as controls on the effectiveness of the surface decontamination procedure. These tubes are capped in a manner which permits the gas phase to enter. At the end of the surface decontamination period, 10 ml of growth medium is added and the tubes still inside the bag are incubated under the same conditions as the assay tubes. The screw capped tubes containing the culture media and the neutralizer are sealed to prevent contact with the ethylene oxide. No evidence of outgrowth has been obtained in those tubes in the tests reported in this study.

C. Analysis of Ethylene Oxide Concentration

Gas samples for the assay of ethylene oxide and humidity were collected using a 10 ml syringe. The needle is introduced into the bag at the site of a double rubber seal. Past experience has shown that leakage from this sampling site does not take place; however, added assurance of protection against this is provided by using pressure sensitive tape over the puncture area. Ethylene oxide escapes from the bag at a very slow rate thus necessitating an additional ethylene oxide analysis at the end of the decontamination period. The humidity and ethylene oxide concentrations are determined by gas chromatography utilizing the same gas chromatographic column (1% carbowax 1540 on Teflon 6) using a gas chromatograph equipped with a thermal conductivity detector (Carle Instruments, Inc.). This instrument can detect 10^{-6} g of ethylene oxide or H_2O/ml at maximum sensitivity.

D. ~~Exhaustion~~ Exhaustion of Ethylene Oxide and Replacement with Air

Following the sterilization cycle, the residual ethylene oxide is exhausted from the bag, and the chamber is flushed 5 times with alternate filling and exhausting sterile air. This is facilitated by using a tube packed with sterile bacteriological cotton which serves to remove gross particles and microorganisms passing through the tubing line from the exterior of the bag. Also in this line, a Millipore filter provides an additional barrier to the contamination of the bag through this line. To further insure that no remaining traces of ethylene oxide are present within the bag, a 10 ml volume of the air within the bag is withdrawn and injected into the gas chromatograph to determine the concentration of ethylene oxide.

E. Inoculation and Pulverization of the Solid

The plastic and hardener are weighed out and mixed according to the manufacturer's directions. The mixing is accomplished under clean conditions. The inoculation of the solid is performed just prior to its solidification. Normally, at this stage the solid has warmed appreciably

due to the exothermic reaction taking place. If the temperature is in excess of 35°C, it is cooled to or below that temperature prior to the addition of the inoculant. After the inoculant is added, it is quickly stirred and tamped in order to release any gas bubbles present within it. After the solid has solidified, it is removed from the mold and subjected to pulverization procedures.

The solid is placed in the vise, the collector is placed beneath the solid and the sample sawing is performed. The pressure of the blade on the solid is of critical importance since it determines to a major extent the thickness of the spiral-shaped ribbons that are formed. The weight of the holder and blade/inch of blade constitutes the only pressure applied during the sawing process. Under ideal circumstances, the pressure should be 100 gm/inch or less. The rate of sawing determines the amount of heat produced at the interface of the blade and the solid (5). The weight of the sample is estimated using a 5 ml beaker. This does not provide sufficient accuracy but allows crude estimations of the quantity of the solid processed. (The exact weight of solid added may be determined at later steps when the bottle containing the solid is subjected to culturing. The tared weight of the bottle and estimation of all subsequent volume permits the estimation of the total weight of the solid sample).

In spite of the fact that hacksaw blades are constructed from high-grade hard steel alloys, they dull at surprisingly fast rates. With epoxy solids, the blade should be changed after the pulverization of 5 grams of solid. The changes observed when pulverization is accomplished with a dull blade are characterized by ribbons of irregular thickness and a tendency of the solid to break into large chips.

Current work indicates that it is easier to disintegrate the solid material if it is subjected to a brief grinding with neutralizer in a mortar and pestle. This grinding is carried out with a pestle chilled by placing a corner of the bag containing the pestle in an ice bath. Exposure of the contents of the mortar and pestle to ultrasound, utilizing a Branson S-75 ultrasound stephorn at an output of 5 amps for thirty seconds, increases the recovery of spores. The bag decreases the efficiency of the ultrasound probe and thus it is necessary to use full output.

F. Neutralization

It is convenient to add the neutralizer immediately following the sawing step (5% RNA, 5% beef extract). The quantity of the neutralizer used in these experiments is 2 ml per gram of solid.

At the end of the grinding and ultrasound period, the sterile culture medium maintained at 40°C by a water bath is added to additional neutralizer (8 ml). The mortar and pestle are withdrawn from the ice bath and allowed to sit for one hour to allow the neutralizers to overcome the growth inhibitory substances present. The contents of the mortar following sufficient mixing to produce a homogeneous mixture are added to a test tube and the test tube closure is sealed. Care must be taken in the latter steps to accomplish the mixing with sufficient rapidity so that the semisolid agar medium-neutralizer mixture does not solidify prematurely.

The adjustment of pH, redox potential, and control of fluidity are performed within the sterilant bag. Model experiments previously performed outside of the bag using the same weight of sample provide the volume requirements for the oxidants, reductants and other such additives. These manipulations prevent contamination by pH meters, volumetric burets and such instrumentation if they were included within the bag.

G. Leaching

Leaching the pulverized solid at 5°C is partially effective in removing growth inhibitors of certain solids. The use of neutralizers as outlined above is generally as effective in overcoming the toxicity of the solid. The use of both neutralizers and leaching steps does not at this time appear to be warranted since the increase in recovery when both procedures are applied is not sufficiently high to be useful.

H. Culturing

The composition of the culture media used in these experiments is shown in Table 9. This growth medium was devised to permit the growth of a wide variety of spore forming microorganisms in addition to B. subtilis.



The culture tubes with the closures tightly in place are incubated at 32°C. With B. subtilis var niger, the maximum number of colonies is observed following an incubation period of three to four weeks.

Nondamaged spores incubated in this culture medium grow out rapidly with maximum number of organisms evident within a period of one to two days. It is suggested that the delayed appearance of colonies is dependent upon chemical and/or physical injury sustained in the incorporation into the solid epoxy resin or in the process of recovery from the resin.

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TABLE I-A

SIZE OF PARTICLES AND INTERVALS BETWEEN CRACKS AND IMPERFECTIONS

(STAR BLADES No. 1218-3 (12 x 1/2 x .025 - 18 teeth)

Plastic	Vertical Pressure (grams) Sawing	% of Particles (Average of 3 Replicates)							
		Interval between cracks and size of particles (microns)							
		<1	1	1-2	2-5	5-10	10-20	20-40	>50
Maraset	19	4	9	11	20	15	26	8	7
	152	2	4	8	22	19	26	10	9
	546	2	3	4	9	13	27	18	24
Stycast	19	5	8	10	19	16	28	9	5
	152	3	5	9	20	22	25	9	7
	546	1	5	7	11	19	30	19	8
Epocast 4D	19	2	5	12	21	17	25	12	6
	152	1	4	7	24	21	26	11	6
	546	2	2	4	13	23	27	20	9
Epocast 4H	19	3	7	11	21	15	28	11	4
	152	1	5	7	21	20	27	10	9
	546	2	4	6	12	22	29	17	10
Epocast 4B	19	1	7	9	23	19	27	11	3
	152	2	5	8	25	17	28	6	9
	546	1	2	5	13	25	28	18	8

TAL I-B

SIZE OF PARTICLES AND INTERVALS BETWEEN CRACKS AND IMPERFECTIONS

(STAR BLADES NO. 1218-3 (12 x 1/2 x .025 - 24 teeth)

% of Particles (Average of 3 Replicates)

Intervals Between Cracks and Size of Particles (microns)

Plastic	Vertical Pressure (grams) Sawings								
		<1	1	1-2	2-5	5-10	10-20	20-40	>50
Maraset	19	17	23	17	11	12	12	4	4
	152	9	14	19	21	17	10	7	3
	546	3	6	10	14	21	12	14	20
Stycast	19	11	13	16	12	19	19	7	3
	152	6	8	9	20	18	20	11	8
	546	3	8	10	16	20	24	11	7
Epocast 4D	19	13	21	18	11	13	9	8	7
	152	10	21	12	15	12	17	6	7
	546	5	11	15	14	17	23	9	6
Epocast 4H	19	9	14	17	10	16	12	7	5
	152	8	10	11	19	17	19	10	6
	546	4	9	12	15	19	23	11	7
Epocast 4B	19	12	22	16	10	16	11	8	5
	152	7	14	11	21	17	18	7	5
	546	4	10	14	14	19	22	10	7

2

TABLE I-C

SIZE OF PARTICLES AND INTERVALS BETWEEN CRACKS AND IMPERFECTIONS

(DISTON BLADE SPECIAL ALLOY DURAFLEX NO. F-1232 (12 x 1/2 x .025 - 32 teeth)

Plastic	Vertical Pressure (Grams Sawing)	% of Particles (Average of 3 Replicates) Intervals Between Cracks, Size of Particles (microns)							
		<1	1	1-2	2-5	5-10	10-20	20-40	>50
Maraset	19	21	26	16	12	6	10	4	4
	152	11	17	21	23	15	6	5	2
	546	2	7	12	15	20	10	12	22
Stycast	19	13	15	17	14	19	11	5	6
	152	7	10	9	19	18	16	13	8
	546	5	9	12	17	21	17	7	12
Epocast 4D	19	19	25	20	12	7	8	3	6
	152	16	26	19	13	11	10	5	9
	546	7	13	23	17	12	12	6	10
Epocast 4H	19	17	20	19	11	15	9	4	5
	152	9	17	15	13	19	15	5	7
	546	5	9	12	9	25	18	10	12
Epocast 4B	19	13	19	19	18	16	6	6	3
	152	9	20	16	12	22	9	7	5
	546	8	12	14	13	19	14	9	11

TABLE 2-A

NEUTRALIZATION OF BENZOYL AND ISOBUTYRYL PEROXIDES

<u>Compound</u>	Percent of Control Counts (Average of 5 plate counts)			
	Sodium Borohydride 0.1M	Cysteine 0.1M	Glutathione 0.1M	Water
Benzoyl Peroxide, 5%	73	52	92	0
Isobutyryl Peroxide, 5%	65	50	88	0
Control (H ₂ O)	100	100	100	100

After reduction of the peroxide, 10^3 spores of B. subtilis var niger were added. After exposure for 2 hours at 25°C, the organisms were counted by plate dilution technique subsequent to incubation at 35°C for 24 hours.

TABLE 2-B

NEUTRALIZATION OF BENZOYL AND ISOBUTYRYL PEROXIDES

<u>Compound</u>	Percent of Control Plates			
	Sodium Borohydride 0.1M	Cysteine 0.1M	Glutathione 0.1M	Water
Benzoyl Peroxide, 5%	12	18	27	0
Isobutyryl Peroxide, 5%	17	20	32	0
Control (H ₂ O)	89	99	100	100

After reduction of the peroxide, 10^3 spores of C1 sporogenes were added. After exposure for 2 hours at 25°C, the organisms were counted by plate dilution technique subsequent to incubation at 35°C for 24 hours.

TABLE 2-C

NEUTRALIZATION OF BENZOYL AND ISOBUTYRYL PEROXIDES

<u>Compound</u>	Percent of Control Plates			
	Sodium Borohydride 0.1M	Cysteine 0.1M	Glutathione 0.1M	Water
Benzoyl Peroxide, 5%	2	3	14	0
Isobutyryl Peroxide, 5%	6	8	16	0
Control (H ₂ O)	50	64	97	100

After reduction of the peroxide, 10^3 cells of E. coli were added. After exposure for 2 hours at 25°C, the organisms were counted by plate dilution technique subsequent to incubation at 35°C for 24 hours.

TABLE 2-D

NEUTRALIZATION OF BENZOYL AND ISOBUTYRYL PEROXIDES

<u>Compounds.</u>	Percent of Control Plates			
	Sodium Borohydride 0.1M	Cysteine 0.1M	Glutathione 0.1M	Water
Benzoyl Peroxide, 5%	47	51	66	1
Isobutyryl Peroxide, 5%	40	47	52	2
Control (H ₂ O)	82	98	100	100

After reduction of the peroxide, 10^3 spores of Ulocladium were added. After exposure for 2 hours at 25°C, the organisms were counted by plate dilution technique subsequent to incubation at 35°C for 24 hours.

TABLE 3

NEUTRALIZATION OF EPICHLORHYDRIN

% of Control (Average of 3 Replicates)

Treatment	<i>B. subtilis</i>	<i>Cl sporogenes</i>	<i>S. marcescens</i>	<i>Ulocladium</i>	<i>M. epidermitidis</i>	<i>E. coli</i>
5 ml H ₂ O	0	0	0	0	0	0
Adjust pH to 3, hold 2 hours, readjust to 7	42	32	12	0	0	0
Adjust pH to 4, hold 4 hours, readjust to 7	28	11	7	0	0	0
Adjust pH to 4, hold 4 hours, presence of 5% histidine, readjust to 7	67	50	20	0	0	0
Adjust pH to 4, hold 15 minutes, readjust to 7	12	7	4	0	0	0

10⁴ organisms were added to a final volume of 3 ml of 5% epichlorhydrin immediately treated as indicated, and brought to a final volume of 5 ml. Recovery of viable organisms was measured as percent of control unexposed to epichlorhydrin. The organisms were counted by plate dilution technique subsequent to incubation at 35°C for 48 hours.

TABLE 4

THE EFFECT OF EPICHLORHYDRIN ON THE AMINO ACIDS OF CASEIN

<u>Amino Acid</u>	<u>AMINO ACID COMPOSITION (%) (Average of 3 Replicates)</u>	
	<u>Before Reaction</u>	<u>After Reaction</u>
Arginine	4.3	4.1
Histidine	2.2	0
Lysine	7.5	7.1
Tyrosine	6.9	6.3
Tryptophane	1.2	0
Phenylalanine	4.8	4.2
Cystine	0.35	0
Methionine	3.4	3.1
Threonine	3.8	2.1
Serine	7.6	5.2
Leucine	9.8	8.8
Isoleucine	6.2	5.7
Valine	6.6	6.2
Glutamic acid	23.3	21.3
Aspartic acid	6.0	5.8
Glycine	0.5	0.2
Alanine	5.5	4.1
Proline	7.5	6.2
Hydroxyproline	0.2	0

Two grams of casein were reacted with 100 ml of 0.5% epichlorhydrin at 20°C for 30 minutes at PH 7.0. Note that all of the histidine was destroyed while very little reaction occurred with the other amino acids.

TABLE 5

THE EFFECT OF VARIOUS NEUTRALIZERS ON THE RECOVERY OF MICROORGANISMS

<u>Neutralizer</u>	<u>% of Control (Average of 5 Replicates)</u>		
	<u>B. subtilis</u>	<u>Cl. sporogenes</u>	<u>S. marcescens</u>
Histidine, 5%	12	8	10
Casein, 5%	3	0	0
Beef Extract, 5%	25	17	21
Beef Extract, 10%	28	24	25
RNA, 5%	22	20	22
RNA, 10%	20	-	19
RNA, 5% and			
Beef Extract, 5%	71	55	61
* RNA 10%, Beef Extract 10%			
Water	0	0	0

10^4 organisms were added to a final volume of 3 ml of epichlorhydrin immediately treated with the neutralizer at 0-5°C in a final volume of 6.0 ml. Recovery of viable organisms was measured as percent of control unexposed to epichlorhydrin. The organisms were counted by plate dilution technique after incubation at 35°C for 48 hours.

TABLE 6

THE EFFECT OF LEACHING ON THE RECOVERY OF ORGANISMS FROM ECCOCOAT 1C2

<u>Leaching Medium</u>	% Recovery (Average of 3 Replicates)							
	<u>B. subtilis</u>		<u>Cl. sporogenes</u>		<u>S. marcescens</u>		<u>Ulocladium</u>	
	5°C	25°C	5°C	25°C	5°C	25°C	5°C	25°C
Water, pH 2	0	0	0	0	0	0	0	0
Water, pH 5	0	0	0	0	0	0	0	0
Water, pH 7	0	0	0	0	0	0	0	0
Ethylene Chloride	3	1	5	2	4	3	0	0
Chloroform	4	1	6	2	5	2	0	0
Acetone	2	0	0	1	1	1	0	0
Trichloro Trifluoro Ethane	5	1	2	0	6	0	0	0
Carbon Tetrachloride	2	0	0	0	0	0	0	0
Acetone: H ₂ O (50:50 vol/vol)	2	2	0	1	2	0	0	0

The organisms were inoculated at a level of 10^3 /gm of solid. The leach fluid 10 ml/gm solid were added to the pulverized Eccocoat, allowed to leach for 2 hours, filtered and cultured on membrane filters. The membrane filter cultures did not show growth and therefore, no values for the leach fluid are shown. The pulverized, leached solid was also cultured and the values reported above. The methods outlined in the protocol section were used for cultural recovery. The cultures were incubated at 35°C. The colonies were counted at 2 week intervals. The counts for these cultures were maximal at 5 weeks of incubation.

TABLE 7
EFFECT OF ULTRASONIC OSCILLATION ON RECOVERY
OF B. SUBTILIS VAR NIGER FROM STYCAST 1090

<u>Time of Exposure</u>	(Average of 3 Replicates) number recovered/gram (247)	<u>% Recovery</u>
0		100
15 secs.		99
30 secs.		118
1 min.		135
2 mins.		144
6 mins.		165
8 mins.		161
10 mins.		169
15 mins.		160
30 mins.		141

TABLE 8

RECOVERY OF MICROORGANISMS FROM SOLIDS

% Recovery (Average of 3 Replicates)

<u>Solid</u>	<u>B. subtilis</u>	<u>Cl. sporogenes</u>	<u>S. marcesens</u>	<u>Ulocladium</u>	<u>M. epidermitidis</u>	<u>E. coli</u>
Eccocoat 1C2	2	4	2	0	0	0
Epon 901	4	4	1	0	0	0
Paraplast	78	70	66	29	16	9
Parlodion	51	66	46	3	0	0
Stycast	32	11	19	0	0	0
Maraset	38	5	22	0	0	0
RTV 40	3	1	5	2	0	0

The organisms were inoculated at a level of 10^3 /gm of solid. The methods outlined in the protocol were used for the cultural recovery. Cl. sporogenes was incubated under anaerobic conditions. The colonies were counted at 2 wk. intervals. The counts for these cultures was maximal at 5 weeks incubation.

TABLE 9
CULTURE MEDIUM

	<u>Per cent Composition</u>
Tryptone	5.0
Casamino acids	0.25
Bacto soytone	0.2
Soluble starch	0.5
Dextrose	1.5
d l - Alanine	0.5
DNA	0.2
Tetrahydrofurane	0.5
Yeast extract	0.9
Magnesium sulfate	0.2
Azolectin	0.25
*Tween 80	0.15
Potassium phosphate dibasic	0.5
Agar	0.8
*Polyoxyethylene sorbitan monooleate	

TABLE 10

LIST OF MATERIALS OF ENCLOSURE AND CONTENTS

1. Ethylene oxide or ethylene oxide-Freon 12 mixture.
2. 4-8 mil polyethylene tubing 50-100 cm width of 50-100 cm length.
3. Screw capped culture tubes, 25 cc capacity
 - a. empty
 - b. containing 10 ml of neutralizer (1 for each solid sample of 1 g).
 - c. containing 10 ml culture media (4 for each solid sample of 1 g).
 - d. control tubes previously inoculated with 10^2 spores of B. subtilis var niger
 - e. same as d but inoculated into 10 ml of growth media.
4. Vise mounted on solid surface
5. Saw blade and holder with total weight on sawing edge of 50-100 g/inch
6. Mortar and pestle. Paper wrapped 125-500 ml capacity (1 for each solid to be assayed).
7. Paper towels - 6
8. 45°C water bath
9. Ice bath
10. Screw capped bottles containing
 - a. 50 ml sterile distilled H₂O
 - b. sterile 0.01 N NaOH
 - c. sterile 0.01 N HCl
 - d. sterile 0.1 M glutathione

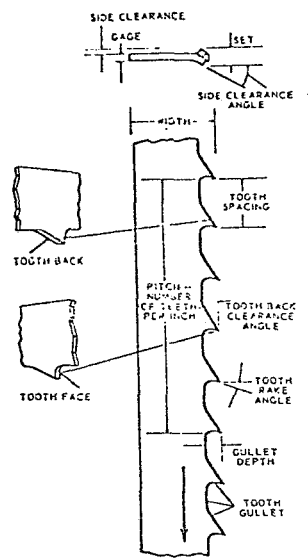


FIGURE 1. The design features of saw blades.

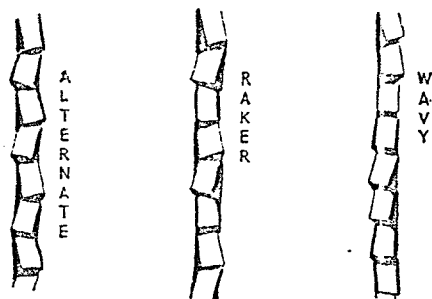


FIGURE 2. Teeth settings of saw blades.

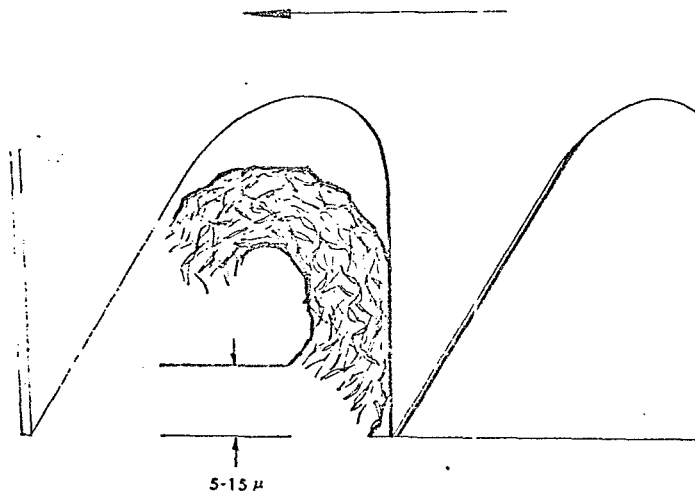


FIGURE 3. Saw - solid interaction.

Large arrow indicates direction of saw movement. In some instances, a ribbon which is highly fractured is produced as shown, whereas, with most brittle solids, fragmentation into small particles occurs.

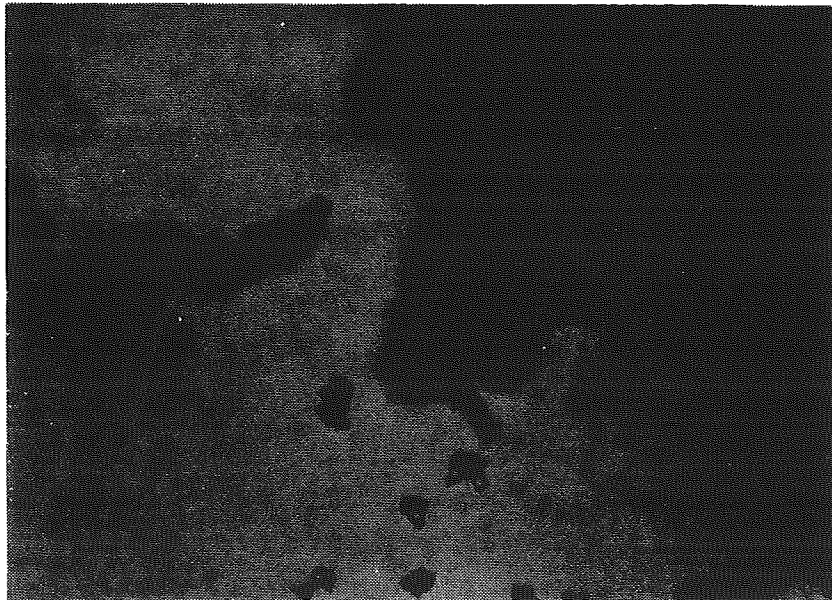


FIGURE 4. Microphotograph of solid rocket propellant.

Note the agglomeration of particles and the presence of fractured ammonium perchlorate crystals.

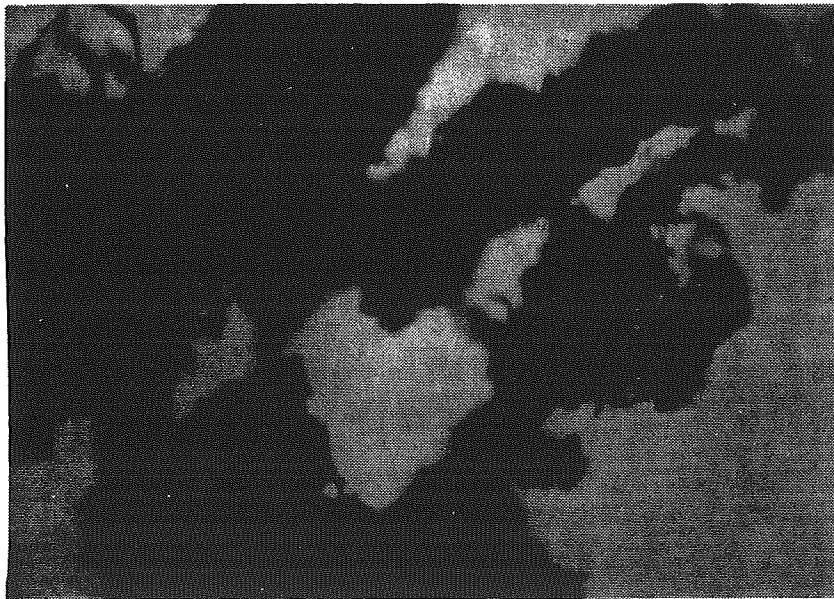


FIGURE 5. Microphotograph of solid rocket propellant in water.

Most crystals of ammonium perchlorate have dissolved,
exposing the binder.

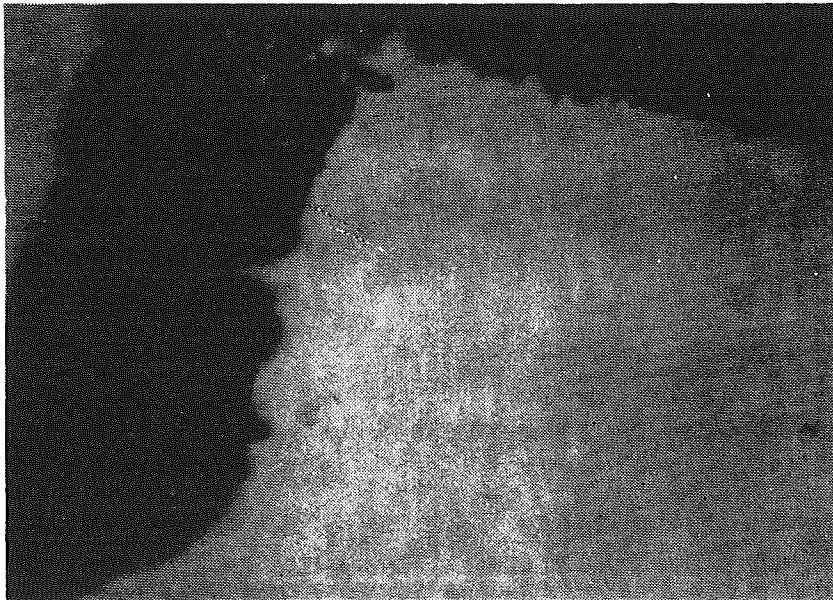


FIGURE 5. Microphotograph of ribbon produced from Epocast 4H.

Note the microcracks, surface irregularities and imperfections within the ribbon.

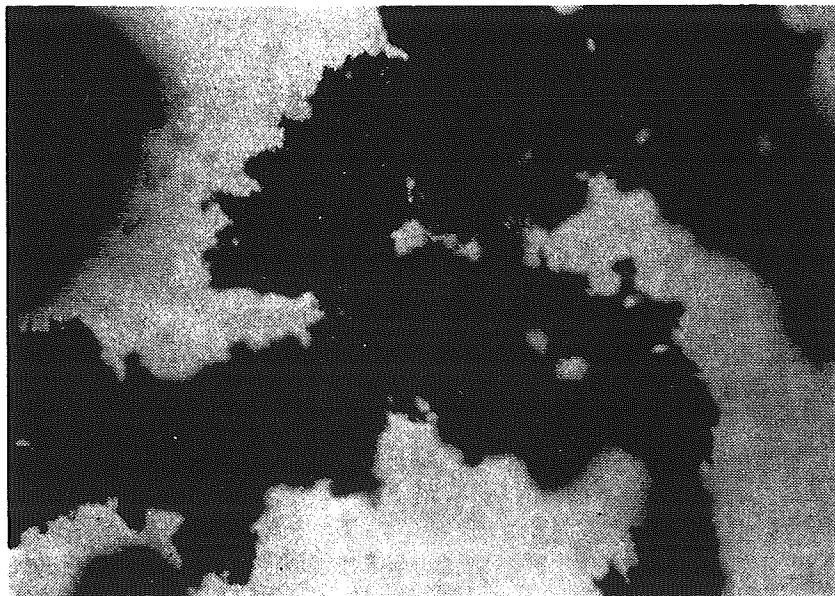


FIGURE 7. Microphotograph of ribbon produced from Epon 901/B1.

Note the microcracks, surface irregularities and imperfections within the ribbon.

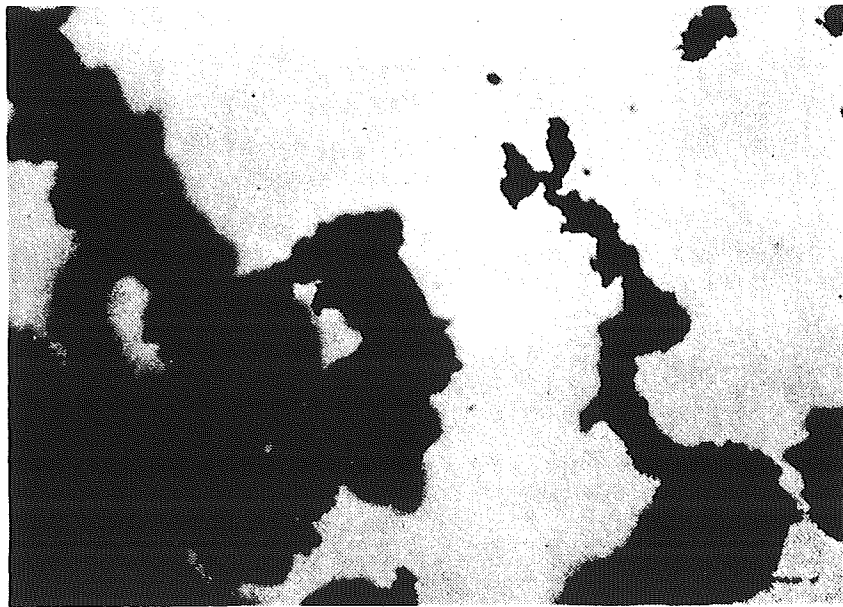


FIGURE 8. Microphotograph of Stycast 1090 ribbons.

Ribbon was produced under 500 grams/inch pressure on the blade. Stycast fragments into smaller particles when sawed at 100 grams pressure, producing a mixture of ribbon fragments and free particles.

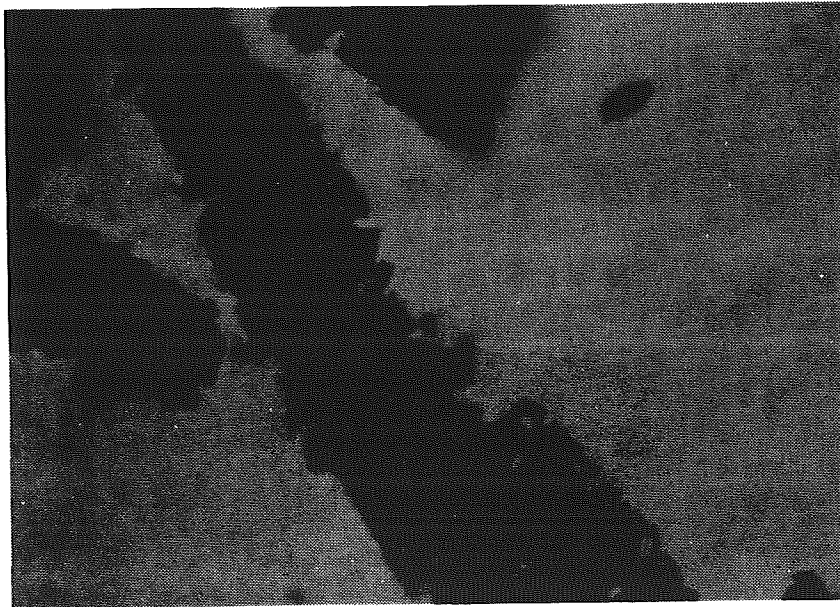


FIGURE 9. Microphotograph of Epocast 212/951.
Note fragments lying on edge.

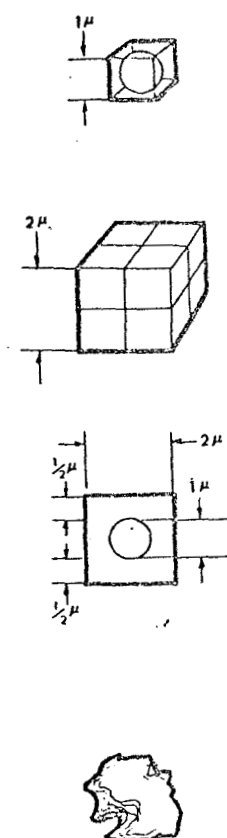
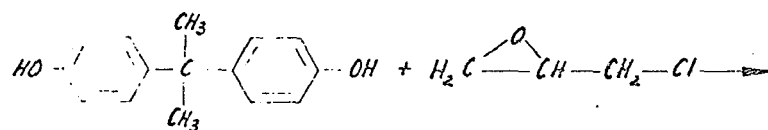


FIGURE 10. Theoretical model of entrapped spore.



BISPHENOL A

EPICHLORHYDRIN

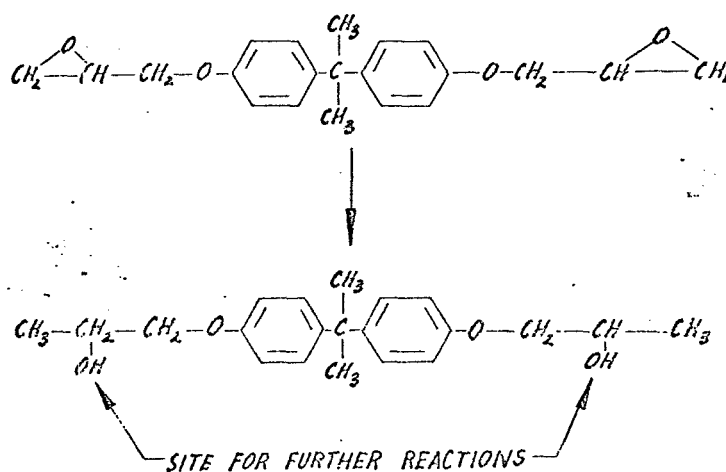


FIGURE 11. Synthesis of epoxy plastics.

THE EFFECT OF EPICHLORHYDRIN AND
EPOXY TYPE COMPOUNDS ON NUCLEIC ACIDS

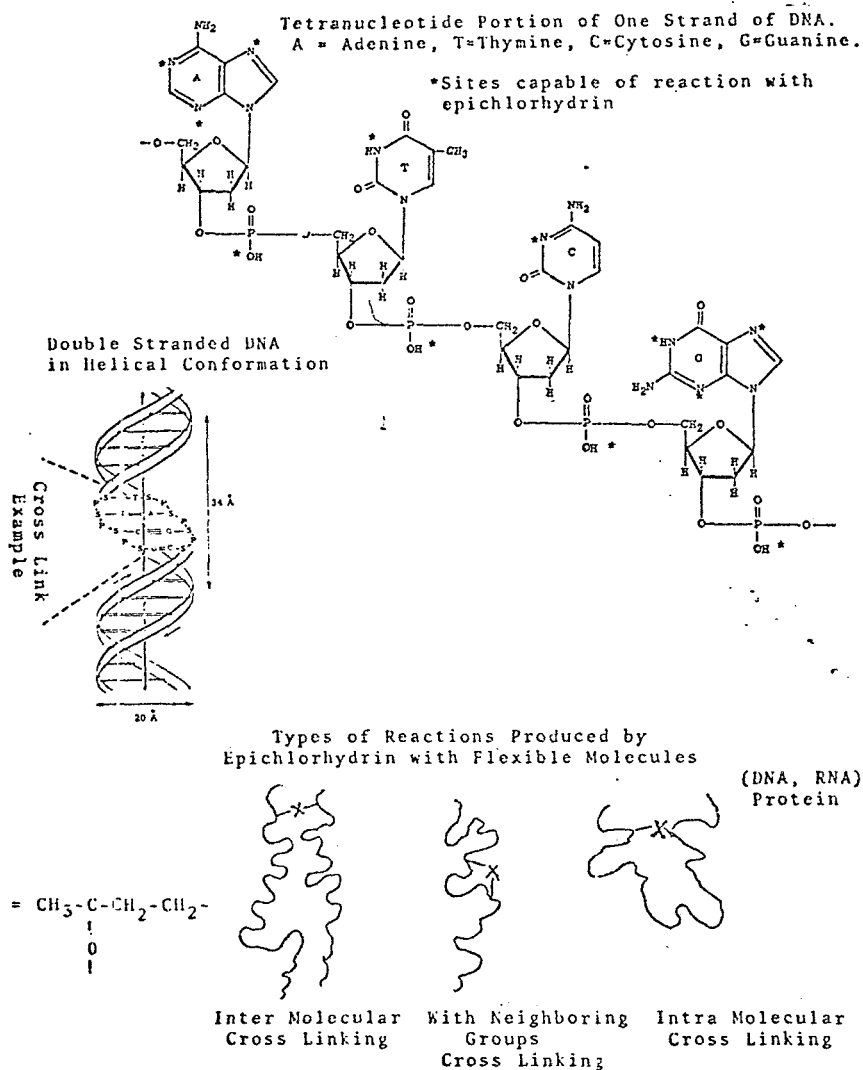


FIGURE 12. Interaction of epichlorohydrin with nucleic acids and proteins.